The Application of Chitosan on an Experimental Infection of Pseudogymnoascus Destructans Increases Survival in Little Brown Bats

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THE APPLICATION OF CHITOSAN ON AN EXPERIMENTAL INFECTION OF
PSEUDOGYMNOASCUS DESTRUCTANS INCREASES
SURVIVAL IN LITTLE BROWN BATS

by

Torin C. Kulhanek

A thesis submitted to the Graduate College
in partial fulfillment of the requirements
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This research builds upon a previous study conducted that tested the efficacy of chitosan on experimentally-infected bats as a laboratory model of White-nose syndrome. White-nose syndrome is a fungal infection caused by *Pseudogymnoascus destructans*, formerly known as *Geomyces destructans*. The pathology of this disease is not yet fully understood, but it is devastating bats throughout Northeastern America and continues to spread. Here we tested multiple application time points and concentrations of chitosan on the wings of experimentally-infected bats and compared them with the controls. We grossly viewed the wings first to get a sense of the amount of damage that was present and found that closer examination was required. We then closely examined the muzzles for the presence of *Pd*. Finally, we closely examined the wings for the presence of an inflammatory response in the bats after arousing from torpor indicated by the presence of neutrophilic infiltrations. Chitosan significantly limited fungal presence in the muzzles and improved the survival outcome of little brown bats. The fungus clearly caused inflammation in WNS-affected bats after emergence from torpor. These data support the use of chitosan for bats with WNS and merits further testing to determine the appropriate dosage amount and delivery methodology for field trials.
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INTRODUCTION

Many hibernating bats in Northeastern America have contracted an emerging disease called White-nose syndrome (WNS), which is caused by the fungus *Pseudogymnoascus destructans* (Pd) (Minnis and Linder 2013), formerly known as *Geomyces destructans* (Lorch et al. 2011). Visibly, WNS causes a white fungal growth on the bats’ muzzles and a white tacky discoloration on their wings and ears (Meteyer et al. 2011). Additionally, it causes skin lesions to develop on their wings (Wibbelt et al. 2013).

*Pd* was first documented in the U.S. in 2006 inside Howes Cave, about 52 km west of Albany, New York (Blehert et al. 2009). However, it likely originated in Europe and was unknowingly introduced as a novel pathogen to North America (Warnecke et al. 2012). This was based on the susceptibility of North American bat species to *Pd*. They also hypothesize that *Pd* could have been possibly introduced to North America by tourists visiting caves from Europe.

Interestingly, European bats did not have high mortality rates as compared to many species of North American bats (Warnecke et al. 2012), and may tolerate the infection, indicating possible host-pathogen equilibrium has been established (Zukal et al. 2016). Phylogenetic results and analyses from Minnis and Lindner (2013) suggest that no closely related sister taxa in hibernacula in eastern North America exist, and this provides further evidence for an exotic invasive hypothesis by *Pd*.

Bats in general are extremely beneficial to ecosystems and have a direct impact on the human condition. They help to control pest populations as many bat species are top insect predators (Brownlee-Bouboulis and Reeder 2013) and play critical roles in controlling some insect populations (Blehert et al. 2009; Reynolds and Barton 2014). Bats also assist in pollination
of flowers and plants (Blehert et al. 2009). However, this emerging disease could have devastating effects (i.e. endangerment and extinction) on many bat populations in North America. The question remains whether anything can be done to successfully combat this disease and prevent it from causing decline in North American bat populations.

There is no cure for this disease currently, but the scientific community has conducted many studies to learn more about it and its causative agent. The scientific community has also attempted to reduce its spread by educating the public on the problem (Foley et al. 2011). It is hoped that by studying this disease and developing suitable treatment options bat populations may be saved in the future. The overall goal of the present study was to understand more about this specific problem and further test a novel treatment option for bats with *Pd* infection.

The overarching problem is that several bat populations in North America are experiencing widespread mortality as a result of *Pd* infection. It has been reported that bat populations in affected hibernacula have declined by greater than 75% (Blehert et al. 2009). The North American little brown bat (*Myotis lucifugus*) is one of the hardest hit species and the northern long-eared (*Myotis septentrionalis*), eastern pipistrelle (*Perimyotis subflavus*), and big brown (*Eptesicus fuscus*) bats are just a few other known affected bat species (Courtin et al. 2010). Prior to 2012, 17 total vespertilionid bat species had been reported positive for *Pd* in North America and Europe, but it is expected that this number will rise (Zukal et al. 2014).

At present, WNS continues to increase in prevalence and expand across North America. It does not appear to be species-, genus-, or family-specific as indicated by the list of known affected species and it has been suggested that *Pd* is a generalist pathogen meaning all hibernating bats within its distribution may be at risk for infection (Zukal et al. 2014). Thus, if no solution is found, then this disease may wipe out multiple bat species in the future.
The morphology of *Pd* is unique as a fungal species. It has filamentous hyphae, but curved conidia (Gargas et al. 2009), which is a distinguishing characteristic of this fungus. *Pd* is psychrophilic, or cold-loving, and can grow anywhere between 5°C and 20°C (Blehert et al. 2009) and grows optimally between 12.5°C and 15.8°C (Verant et al. 2012). It grows radially and reproduces asexually, like other fungi, forming conidia at the end of long and branched conidiophores (Verant et al. 2012; Gargas et al. 2009). Additionally, it grows slowly on artificial media and does not grow at 24°C or above (Gargas et al. 2009). Figure 1 below shows an example from the current study of the morphology of *Pd* using periodic acid-Schiff staining.

Figure 1. Morphology of *Pseudogymnoascus destructans*
Smaller round conidia and larger long, filamentous hyphae are both present in the above image. It stains relatively-well using this method showing a dark magenta color. Blurriness was caused by multiple layers of hyphae and conidia atop each other.

The known pathogenesis of *Pd* is also unique. Bats enter short periods of low physical and metabolic activity called torpor during the hibernation season to conserve energy (Bouma et al. 2010). The fungus affects hibernating bats by invading deeply into their subcutaneous tissue, leading to ulcerative necrotic spots and destruction of wing membranes (Meteyer et al. 2009). Generally, an animal’s immune system can keep a fungus present on the skin subclinical during non-hibernating seasons. However, there is a reduction in the immune systems of hibernating bats (Bouma et al. 2010), which means a fungal pathogen can easily infect them. Without an active immune system, *Pd* is able to colonize and erode the skin of bat hosts (Meteyer et al. 2012). Between 8°C and 14°C describes the temperature range of caves where bats and other animals hibernate (Bouma et al. 2010) and little browns specifically will hibernate between <10°C and -2°C (Carey et al. 2003; Heldmaier et al. 2004). Thus, *Pd* is suited for this type of environment.

The fungal infection begins growing on the surface of the skin (i.e. stratum corneum) and hair, and eventually penetrates through the epidermis causing further damage, especially in the wings. Areas of the body that the fungus typically affects are the muzzles, wing membranes, and the pinnae of the ears of bats (Gargas et al. 2009). Ulceration is a common occurrence as the fungal invasion destroys the epidermal basement membrane throughout the wing (Wibbelt et al. 2013). Fungal hyphae invade hair follicles and their associated glands, destroying the regional tissue (Blehert et al. 2009). They erode and replace the living skin of bats during hibernation, but
is usually limited to the skin (Cryan et al. 2010). A previous study reported no evidence of the disease found in the internal organs of bats (Wibbelt et al. 2013).

The way in which *Pd* affects a bat’s immune system is highly important for better understanding the pathology of this disease. As mentioned earlier, there is a reduction in the immune system, notably the innate immune system, during hibernation in bats as the body temperature drops to help conserve energy (Bouma et al. 2010). This could explain why the fungus does not typically cause an immune response in the tissues of hibernating bats (Meteyer et al. 2009). While microbes generally cannot proliferate at lower temperatures, *Pd* could be an exception as it is a psychrophilic fungus (Bouma et al. 2010). It continues to invade the tissue while there is an absence of the inflammatory response in hibernating bats; however, neutrophil granulocytes can be occasionally observed in the regional connective tissue (Meteyer et al. 2009). Still, with little to no immune function, hibernating bats are a very suitable host for the fungus to infect as they provide nutrients and an ideal environment (Cryan et al. 2010).

When a bat emerges from torpor, its immune system returns to normal functioning levels and mature neutrophil numbers will increase in response to existing *Pd* infections (Bouma et al. 2010). This rise in circulating neutrophils is the result of increased maturation of neutrophilic band cells, or release of retained, mature neutrophils in sequestered areas of the body, such as the bone marrow (Bouma et al. 2010). However, it is evident that this may not be enough for an infected bat to fight off WNS. Returning to euthermic temperature levels is energetically costly for bats emerging from torpor and animals already in this weakened state attempting to fight off an established infection may add to the high mortality rates seen in WNS.

Understanding how the disease affects the physiology, and in turn the behavior, of bats is important as well. Hibernating bats store energy in the form of fat for winter because prey is in
limited supply at this time and the bats have little opportunity to restore their energy (Ehlman et al. 2013). Thus, before entering torpor for the winter season, bats must accumulate fat stores from insects (Cryan et al. 2010). *Myotis lucifugus* found in the northern area of their normal range hibernate for about 193 days (Thomas et al. 1990). Normal periods of arousals from bouts of torpor during this hibernating period are highly costly to the bats regarding energy expense because they have to raise their body temperature and metabolic rates to euthermic levels (Thomas et al. 1990). Moreover, these normal arousal periods account for about 1% of the total time spent during winter, but about 80-90% of a bat’s stored energy is consumed during the arousals from torpor (Thomas et al. 1990). Once infected by WNS, bats are drained of their fat reserves prematurely (Verant et al. 2014) as they are aroused from torpor more often than normal (Ehlman et al. 2013) leading to starvation and death. Therefore, each additional arousal beyond the normal periodic ones shortens the hibernation time for a bat by about nine days (Warnecke et al. 2012). It is also believed that severe injury to the wings provokes increased arousal in bats with shortened torpor bouts (Warnecke et al. 2012) and this contributes to mortality (Meteyer et al. 2012) as their energy is depleted. Hibernating little brown bats with WNS have shown to utilize double the amount of energy than non-infected bats under the same experimental conditions, albeit this was not associated with increased rate or duration of arousals (Verant et al. 2014). This may imply that bats with WNS could have increased metabolisms prior to the onset of altered arousal patterns observed later in the infection (Warnecke et al. 2012).

It has been suggested that *Pd* can also irritate a bat’s skin leading to an increased chance of arousal from torpor and subsequent grooming and licking of their wings (Brownlee-Bouboulis and Reeder 2013) and this contributes to energy expenditure and depletion in bats. Brownlee-Bouboulis and Reeder (2013) also found that WNS-affected bats had significantly altered their
behavior during the bouts of arousal as compared to the unaffected ones. That is, bats that remain active for longer periods during the hibernation season will likely have costlier energetic consequences as the authors describe. Fat and energy reserves are crucial for successful hibernation in animals (Blehert et al. 2009) and without them, their chances of survival decline significantly. Therefore, if a bat’s energy reserves are diminished during hibernation, it will likely die, but if it does happen to survive the winter, the infection could leave it with less energy available for reproduction and less able to make it through the next following winter (Ehlman et al. 2013).

Bats might also arouse more frequently in order to attempt to replenish their fluid levels by drinking water and eating snow (Brownlee-Bouboulis and Reeder 2013). Evaporative water loss has been suggested as playing a role in arousal behavior and energy expenditure seen in infected bats (Ehlman et al. 2013) and mortality (Willis et al. 2011). If an affected bat’s blood volume has decreased during torpor, then arousal may result in an attempt to replenish its water levels, but this will lead to an increased energy need and could cause death in an already weakened individual. Moreover, it is possible that the fungal infection is a large contributor to death by causing abnormal water level depletion, increased arousal activity, and rapid energy depletion. Willis et al. (2011) presented findings that support Cryan et al.’s (2010) dehydration hypothesis that bat species (e.g., Myotis lucifigus) already susceptible to dehydration during hibernation should be the most affected by WNS. The dehydration and emaciation together may account for the eventual death of hibernating bats (Wibbelt et al. 2013).

There is also evidence that supports water and electrolyte imbalance, reduction in blood supply to tissues like the wings, and acid-base balance contribute to morbidity and mortality seen in WNS (Warnecke et al. 2013). It is the proper levels of electrolytes that helps maintain
physiological homeostasis (Cryan et al. 2013). Furthermore, when electrolytes, such as Na\(^+\) and Cl\(^-\), are lost during hibernation from WNS, bats may have a difficult time replenishing them because insects are unavailable at this time.

Previous studies have focused a lot on the wings for examination of this infection. This was largely because of how important the wings are for a bat’s survival in terms of maintaining homeostasis. The wings are disproportionately large areas of thin and exposed skin tissue that allow a bat to fly and forage for food (Cryan et al. 2010). Unfortunately, this leaves them vulnerable to such an infection. In healthy bats, the wings are flexible and elastic while contracted and extended (Cryan et al. 2010). However, \(Pd\) disrupts their unique physiological functions by penetrating and invading the dermis (Pikula et al. 2012) that leads to tissue infarction and necrosis, and the disruption of physiological homeostasis thought to be caused by \(Pd\) alone might be sufficient to lead to death (Cryan et al. 2010). Consequently, once the bats no longer have proper control over their physiology, they cannot survive because of starvation.

Destruction of the epithelium in infected areas of the wings likely increases the rate of heat loss from the body, which places a greater energetic cost on re-warming during arousals leading to more rapid depletion of fat reserves (Cryan et al. 2010). Adding to this, the hyphae of \(Pd\) destroy the wing epithelium, including the sebaceous glands that are responsible for waterproofing the wings through secretions (Cryan et al. 2010). If the wings lose their ability to regulate water balance, then bats may be forced to arouse from torpor more often.

Before flight ability is impaired, other abnormal behaviors can be observed in affected colonies that include bats flying out of their caves during daylight hours and wintertime (Courtin et al. 2010). Once the fungus heavily damages a bat’s wings, its flight ability is compromised along with its chances of foraging for nutrients to sustain survival (Reichard and Kunz 2009).
After the fungus has penetrated the vulnerable wing epithelium, flight control and wing stabilization are severely compromised, and nerve damage is very costly to the bats (Cryan et al. 2010). Consequently, a bat without the use of its wings has a very low chance of survival.

Long-term effects of all diseases are crucial in understanding how the future of an affected-species may turn out. WNS may inflict long-lasting injuries to the wings of little brown bats (Reichard and Kunz 2009). It has been noted that many bats emigrating from WNS-affected hibernacula show varying degrees of damage to their wing membranes including scarring, necrosis, and atrophy. Furthermore, lasting wounds or infections on the wings could very much impact a bat’s foraging success after surviving WNS, making it a possible threat even in the active season (Reichard and Kunz 2009). The authors explained that increased severity of wing damage seemed to be associated with poorer body condition. Thus, foraging success is likely affected in these bats. They also suggest that most of the wounds and scars observed on bats during the summer are a direct consequence of *Pd* exposure. That is, when affected bats are forced out of their hibernacula during the wintertime, they may encounter frostbite leading to tissue necrosis because they are not fit for subfreezing temperatures (Reichard and Kunz 2009). They may also collide with trees, rocks, or buildings adding to their risk of injury. It is important to note that wing damage can be the result of many other causes other than WNS. Moreover, distinguishing where visible wing damage arose from is important for determining a treatment’s efficacy.

The spread of any disease is a huge concern in general and understanding it is important for preventing further spread and subsequent problems. WNS is no exception and because bats live in close proximity to one another, the pathogen can easily spread throughout a cave. That is, bats tend to cluster together in cold and high-humidity locations of hibernacula, which could
make them very susceptible to infection by \textit{Pd} and bat-to-bat transmission of it (Ehlman et al. 2013). One study by Lorch et al. (2011) demonstrated that WNS could easily be transmitted from infected bats to healthy ones via direct contact, such as during mating (Wibbelt et al. 2013). This means that the disease can spread rapidly during hibernation as bats cluster together closely and even with other species of bats (Lorch et al. 2011). Additionally, the disease can also spread rapidly just before hibernation season during the swarming process when there is a lot of direct contact between individuals. The authors also tested if the fungus could spread between bats through the air, and determined there was no evidence that supported this. However, the conditions of the incubators used in the experiment might not have been conducive to airborne transfer of conidia.

The specific problem this research focused on is that many bats populations are declining in North America because they usually cannot overcome the infection on their own. There have been ways that others have tested to help save bats infected with this fungus. Multiple traditional synthetic antifungals (e.g. azoles) and biocides (e.g. digitonin) have been shown to have limiting effects on \textit{Pd} (Chaturvedi et al. 2011). However, they have significant drawbacks, such as toxicity to the bats or even their surrounding environment, including other animals.

Previous studies have tested other possible treatments and shown positive results. A study by Boire et al. (2016) used a relatively non-toxic substance known as cold-pressed, terpeneless orange oil, which could inhibit \textit{Pd in vitro}, but additional testing will be needed to assess any undesirable effects on the bats as well as other members of their ecosystems. Cornelison et al. (2014a) tested six bacterially-produced volatile compounds for inhibition of \textit{Pd} and demonstrated that all of them could inhibit growth from conidia and radial mycelial extension. Cornelison et al. (2014b) demonstrated contact-independent antagonism of \textit{Pd} by using an
induced strain of the bacteria *Rhodococcus rhodochrous* could inhibit *Pd* conidia growth successfully at 15°C and had a strong fungistatic effect at 4°C. Hoyt et al. (2015a) isolated six bacteria strains from the skin bats and showed that they could all significantly inhibit the growth of *Pd in vitro*. Zhang et al. (2015) isolated a novel strain of the fungus *Trichoderma polysporum* from a WNS-affected cave and showed that it had specific inhibitory activity against *Pd* in laboratory media and autoclaved soil as a biocontrol agent. Finally, Raudabaugh and Miller (2015) demonstrated the use of a quorum-sensing compound called trans, trans-farnesol, which is produced by *Candida* species, as a way to inhibit the growth of *Pd*. They show how this compound could prevent *in vitro* conidial germination and inhibited growth of preexisting hyphae of *Pd*. While these studies are important for obtaining new knowledge about *Pd*, no treatments have been developed and tested extensively on infected bats in the field yet.

One alternative and novel treatment method is the microbial-static agent known as chitosan. Chitosan is a polysaccharide made up of copolymers of glucosamine and N-acetylglucosamine derived from chitin, which comes from crustacean shells (Ilum 1998). Chitin is naturally abundant throughout nature, such as in the supporting material of insects (Kumar 2000). Unlike chitin, chitosan is water-soluble in acidic media, or in neutral media under precisely specified conditions, opening it up to potential development as a solution and hydrogel (Rinaudo 2006). There are multiple ways that chitosan can be used for, including as a weight-loss aid and cholesterol-lowering agent in humans (Illum 1998). It has many beneficial attributes that include being biocompatible, biodegradable, and non-toxic (Kumar 2000). The most promising developments of chitosan are in the pharmaceutical and biological areas, such as the use of its healing abilities (Rinaudo 2006).
A previous study found that chitosan could significantly limit \textit{Pd in vitro} (Vonhof et al. 2014). It was demonstrated that a 1:10 dilution of chitosan had a strong limiting effect on the growth and germination of the fungus at 84\% and 96\% respectively. Interestingly, they also tested 1\% chitosan on \textit{Pd}-infected bats and observed significantly lower fungal loads and less tissue damage on them compared to controls according to their qPCR results. Therefore, the previous research demonstrated that chitosan had a strong inhibitory effect on the growth of \textit{Pd} both in plate assays and \textit{in vivo} on experimentally-infected bats. However, the initial experiments require follow-up because of inconclusive mortality data, and it is important to understand how chitosan concentration may affect treatment outcomes from both biological and economic perspectives. Additionally, further experiments are required to test how experimentally-infected bats respond to different concentrations of chitosan treatment.

The present study aimed to provide further evidence in support of this treatment on laboratory-infected little brown bats, and is an important next step in the development of chitosan as a viable treatment for bats and bat hibernacula. The overarching hypothesis that this study addressed was to determine if multiple concentrations of the microbial-static agent chitosan could help little brown bats survive a laboratory \textit{Pd} infection.
METHODS

Collection and Organization of Animals for Inoculation Experiment

The study began with the collection of bats from the Iron Mountain Iron Mine located near Vulcan, MI soon after they entered hibernation in early October. A total of 180 North American little brown (Myotis lucifugus) bats were required for the study. Bats were collected from both the ceiling and walls of their hibernacula and placed in portable refrigerators to maintain appropriate hibernation temperatures. There were seven experimental treatment groups and two control groups with 20 bats per group. It was attempted to maintain a 1:1 sex ratio for all groups. The skin of each bat was swabbed and tested for any presence of Pd by quantitative polymerase chain reaction (qPCR) to confirm that they were not infected prior to experimentation. All animals were transported directly from the field to the research facility at Western Michigan University, Kalamazoo, MI, by the PIs. They were housed in a certified BSL2 that required investigators to pass through a changing area to avoid contamination.

1% chitosan in water was the original concentration and was then further diluted for the experimental groups. In the previous experiment, it was found that a 0.1% concentration of chitosan strongly limited Pd in vitro. Therefore, two additional dilutions representing half, 1:20 (0.05%), and twice, 1:5 (0.2%) were tested alongside the 0.1% concentration again. One final concentration that consisted of the original 1% chitosan was also included with the Early Treatment groups.

The first three concentrations had two different time-points of the chitosan application. In the first three treatment groups (Active) bats received an application of chitosan at the beginning of the hibernation season, were allowed to remain active and groom themselves for one day, and
were subsequently inoculated with *Pd* before entering torpor. This simulated concurrent
treatment and exposure that might occur if bats were treated while swarming. In the second three
treatment groups (Early) bats were inoculated with *Pd* at the beginning of the hibernation season,
and chitosan was applied to them 30 days after they had entered torpor as it was tested in the
previous experiment. Alongside the experimental groups, there were two control groups. A
Clean Control group that did not receive any inoculation with *Pd* nor chitosan treatment, and an
Infected Control group that only received inoculation with *Pd*. The final group of 1% chitosan
was an Early-timed treatment group. Solution and chitosan control groups were not required
because the previous research had demonstrated a lack of negative effects of chitosan to healthy
bats, and no effect of the solution treatment relative to infected controls.

All groups were monitored for arousal behavior (frequency and duration) through video
monitoring and temperature data loggers on the bats. At the end of the experiment, body mass
index, degree of fungal presence in the skin, and the degree of tissue damage were recorded. The
outcome of these experiments were intended to clearly indicate if the chitosan concentrations
were efficient at limiting the presence of *Pd* and any associated damage to the skin of bats, as
well as minimizing the length and/or number of arousals and associated mortality.

Experimental Inoculations (*in vivo*)

In total, there were seven experimental treatment groups and two control groups with 20
bats in each. The experimental and control groups were all ran for 150 days in total, which
mimicked the normal hibernation period for bats in the northern Midwest. Both wings were
treated with chitosan in all of the experimental groups. Only the right wing was inoculated with
*Pd* in the Infected Control and treatment groups. The muzzles were neither inoculated nor treated
in any group. Once all animals were collected and transported to Western Michigan University, they were sexed, weighed, had their forearm length measured, and were outfitted with individually-labeled aluminum bands for identification. Each bat was also swabbed to assess *Pd* infection status at the beginning of experimentation.

After processing all bats remained at room temperature for a period of 24 hours in the same wire mesh cages that they were housed in while in the incubators. They were hand-fed with mealworms and watered with an eyedropper following processing and treatment (if applicable).

Processed bats were randomly assigned to experimental treatment and control groups while keeping the ratio of males and females consistent among groups, and placed in cages (one cage per group). Seven experimental groups were divided into two sets of three and four groups based on the time point of treatment, plus two control groups. Three groups of 20 bats were in the Active Treatments, which received an application of chitosan (0.2%, 0.1%, or 0.05% chitosan) immediately following processing on Day 1 of the experiment and were inoculated with *Pd* on Day 2. A different set of four groups of 20 bats were in the Early Treatments and were inoculated with *Pd* on Day 2 of the experiment, but did not have chitosan applications (1%, 0.2%, 0.1%, and 0.05% chitosan) until Day 30. The Infected Control group was inoculated with *Pd* on Day 2 and left untreated. The Clean Control group was not inoculated with *Pd* and did not receive any chitosan application. Any bats not inoculated or treated with chitosan at a particular time-point were sham treated to continue consistent handling among treatments.

The groups were denoted as: Group 1 (Clean Control); Group 2 (Infected Control); Group 3: (Active 0.2%); Group 4 (Active 0.1%); Group 5 (Active 0.05%); Group 6 (Early 0.2%); Group 7 (Early 0.1%); Group 8 (Early 0.05%); and Group 9 (Early 1%).
On Day 1, after processing was completed, bats in the Active Treatments received their respective chitosan treatment. Following the previous research, there was a plastic tub containing excess chitosan into which the extended wing of the bat was dipped into. Both wings were treated in turn, and any excess chitosan solution was able to drip back into the container. Bats in all other experimental and control groups were sham treated by holding them over an empty container and extending their wings.

On Day 2, all bats (except those in the Clean Control group) were then inoculated with *Pd* prepared beforehand using the following procedure. A low-passage isolate of *Pd* from a WNS-positive bat was grown on SDA plates with antibiotics for approximately three weeks. Once sporulation was confirmed, fungal colonies were scraped from the plates into sterile, distilled water. The suspension of fungal elements was filtered through glass wool and the filtrate was centrifuged into pellet spores. The spores were re-suspended in 10 ml of distilled water. They were counted using a hemocytometer and the spore concentration of the solution was standardized for all treatments. On Day 2, using a procedure by Lorch et al. (2011), one 20 μl aliquot of the conidial suspension containing $5 \times 10^5$ spores was then pipetted directly onto the surface of the right wing of the bats.

Following inoculation on Day 2, all bats were transported into artificial hibernacula (incubators) set at a temperature of 6°C and a relative humidity of 98%. The cage containing the Clean Control bats was placed in an incubator that was separate from all remaining bats that were inoculated with *Pd* spores. This prevented any spread of the fungus to the Clean Control bats. Humidity and temperature loggers in each incubator sent data to a wireless router that sent a text message to the investigators’ cell phones if temperature or humidity was not maintained in
the appropriate range. Video cameras containing motion detectors were utilized to record bat movement and behavior and provided a means of monitoring bats throughout the experiment.

On Day 30 of the experiment, all bats were temporarily removed from the incubators. The Early Treatment bats received their respective applications of chitosan, and all other bats were sham treated. All bats were then returned to the incubators for the duration of the experiment (until Day 150).

Video images were monitored twice daily to assess any morbidity and mortality. Dead bats were often difficult to identify in a timely manner. However, any bats that were isolated from the cluster were examined if they did not come out of torpor on an expected schedule. Any bats that appeared moribund, listless or demonstrated outward signs of discomfort or pain (e.g. vocalization or abnormal behavior observed beyond normal movement in the hibernaculum) were euthanized immediately. At the end of the study (150 days) all remaining bats were euthanized using an overdose of isoflurane gas once initial imaging was complete (explained below). They were monitored for cessation of breathing and heartbeat for five minutes or more. At that time, cervical dislocation was utilized to ensure all bats were dead before any postmortem examinations were performed.

The postmortem examination was conducted on all euthanized bats including assessment of grossly visible fungal presence, body mass index, fat levels, and sample collection for histopathology and culture. Wing and muzzle tissues were examined by histopathology with particular attention to the skin. These bats were fixed in 10% neutral buffered formalin for 48 hours before they were processed for histology.

The presence and relative quantity of *Pd* on the tissues was confirmed via qPCR using the most recent standardized protocol (Muller et al. 2013). The qPCR on swabs collected at the
beginning of the experiment was run to confirm infection status of all bats immediately after capture, and at the end to quantify relative fungal loads in response to the experimental treatments.

Harvest

All bats from all groups were harvested on the same day. After they were awoken from torpor, the bats that survived up to the harvest were imaged (see below) and then swabbed, weighed, and euthanized via isoflurane gas. All dead bats were also recorded and prepared for preservation.

Gross Imaging of the Wings

Living bats were imaged in ventral recumbency, with the dorsum of the wing facing up, and the wing held in a stretched open position. A Nikon D3200 digital camera was used to image the dorsal side of each living bat’s right wing above a backlit screen and subsequently under an ultraviolet (UV) light to detect fungal lesions as they fluoresce orange-yellow when hit with long-wave (wavelength 366-385 nm) UV light (Turner et al. 2014). A PeriScan PIM 3 Laser-Doppler System scanner provided by MPI Research was used to image the dorsal side of each living bat’s right wing to detect thermal activity.

Preservation

All bats from all groups were placed in 10% neutral buffered formalin solution for initial fixation inside individually-labeled containers for approximately 48 hours. Afterwards, all bats
were transferred into 70% ethanol for approximately one week and then replaced with new 70% ethanol for continued preservation until sectioning and staining.

Gross Examination of the Wings

Photos of the wings from the digital camera and from the Laser-Doppler System scanner were examined and analyzed using a Dell desktop computer and monitor. The data from the Laser-Doppler scanner were exported to a Microsoft Excel file.

The backlight and UV light images of each bat were closely examined for fungal and lesion presence as well as degree of tissue damage. The backlight images were also used to look at the vasculature diameter in the wings.

The degree of lesion presence on the tissue was scored using a ‘0-3 system’ and closely observing the photos of the wings from the digital camera. Other studies have used similar scoring systems for determining wing damage and histopathology including Meteyer et al. (2011), Reeder et al. (2012), and Reichard and Kunz (2009). A lesion was defined as a noticeable tear (lipping of the epithelium) both full and partial. Any small, well circumscribed smooth bordered full holes or tears near the I.D. band were not included. This was because these tears were thought to be caused by tension from the band rather than the fungus. Additionally, they did not show any evidence of UV fluorescence. The region scored was the plagiopatagium of the right wing as bats were imaged with the dorsum of the wing facing upwards and held in a stretched open position.

A score of ‘0’ indicated no identifiable lesions were present. A score of ‘1’ indicated less than 1/3 of the wing area had identifiable lesions. A score of ‘2’ indicated between 1/3-2/3 of the
wing area had identifiable lesions. Finally, a score of ‘3’ indicated greater than 2/3 of the wing area had identifiable lesions.

UV light pictures were taken along with the backlight images as described above to help further identify evidence of Pd presence. UV fluorescence was used to confirm that lesions were caused by fungal presence by correlating a lesion with fluorescence. It also helped to identify areas that had fungal presence, but no identifiable lesions on backlight images. While these areas did not have a tear on the backlight images, they did have a darker, discolored appearance suggesting that it might be in the early stages of damage. The fluoresced areas were examined to distinguish whether a hyphae pattern or dust pattern was present. If a damaged area did not have the standard characteristics such as the epithelial lipping and irregular border, and there was no support with UV fluorescence, then the area was not considered a lesion. Therefore, it was not included in the scoring.

Alternatively, another idea that was considered for a scoring methodology was using a grid method where a certain number of squares would be overlaid on the images, and each square would get a score of ‘Yes’ or ‘No’ on whether or not it had any evidence of a lesion. Unfortunately, MetaMorph nor the Nikon Elements Analysis program had a feature that could make a grid to meet the demands of the study. Without proper calibration and measurements, the size of the boxes was not known.

There were two ways used to determine the vasculature dilation using the backlight images. The chosen way was by measuring the diameter of the vessels using the main vessel that traveled in about a 45-degree angle across the wing, starting around the elbow and traveling toward the 5th phalange. This vessel was selected because it was consistent throughout all of the bats and it was the largest. A 19445.77 x 19445.77 micrometer square was used to get a sample
area of the main vessel in each bat. It was placed above the elbow, where the vessel intersected the bottom right corner of the box. Five measurements of diameter were taken of the main vessel, dispersed as evenly as possible and then averaged. This was tested on the Clean Control group and Infected Control group because these two groups should have had the greatest difference in vasculature diameter if it truly correlated with infection.

Another way to measure dilation was done so by using the area of the vessels. This could be done in Metamorph by using the threshold feature, but there were many factors that were believed to make this potentially too variable and lead to large amounts of error. For instance, the variation in vasculature color made it hard to distinguish tissue from vessel. Some vessels were too faint where it would be impossible to obtain a threshold. In addition, the amount a vessel would respond to the threshold measurement depended heavily on location and number of clicks on the vessels. There was no pattern that a certain wing region would have increased perfusion. This was true between different bats as well as the same bat. Furthermore, this methodology was not used for any statistical tests.

For examining perfusion in the wings, each living bat’s right wing was scanned under the Laser-Doppler System scanner and the data were viewed in an Excel file. The averages of the perfusion scans in each bat were used to determine any correlation between fungal presence and blood activity in the wings.

Sectioning and Staining of the Muzzles

All bats from all groups were sent off to the Investigative Histology Laboratory at Michigan State University for muzzle sectioning and staining. The muzzles were placed onto a
cutting surface so that the bottom jaw was flat. All muzzles were prepared using the methodology described below in Figure 2.

![Muzzle Sectioning Methodology](Diagram courtesy of Amy Porter, Michigan State University)

The head was held gently with the ears pulled back. Sections were cut every 2.5 to 3.0 mm from the tip of the muzzle to just behind the eyes and in front of the ears. Section 1 tip was embedded on the back side of the cut and sections 2 through 4 were embedded on the front side of the cut. The black lines represent the actual sections of the tissue taken from each bat. Each included animal’s muzzle sections were stained with both period acid-Schiff (PAS) and hematoxylin and eosin (H&E).

**Sectioning and Staining of the Wings**

Living bats from both control groups and the Early 0.1% group were also prepared at the histology laboratory (Michigan State University) for wing sectioning and staining. The Early
0.1% group had the highest survival rate after the Clean Control group. Therefore, the wings from these animals may have had lower amounts of inflammation overall compared to the Infected Control group assuming that fungal presence correlates with an inflammatory response. All wings were prepared using the methodology described below in Figure 3.

Figure 3. Wing Sectioning Methodology
(Photos courtesy of Amy Porter, Michigan State University)

Initially, a wax block was created to pin each bat onto and a moist biopsy sponge was placed on top of it in panels A and B, respectively. The section lens paper was laid over the top of the moist sponge in panel C. Each bat was placed in the prone position with the left wing
opened and pinned to the wax block over the sponge on the inside of center section bones in panel D. Another section of lens paper was placed over the wing followed by a second moist biopsy sponge in panel E. A scalpel was used to cut from the bottom of the wing following along the inside of the bone to the tip of the wing on both sides in panel F. This was done gently to prevent tearing of the tissue. The pins and bat were removed carefully from around the sponges in panel G. Each bat was individually placed into a tissue-processing cassette with the identifying number and closed in panel H. For the embedding procedure, the wing was removed from in between the lens paper and sponges and sectioned using a razor blade into 0.5 to 0.8 mm segments from the outer edge to the tip of the wing in panel I. The sections were then placed onto the edge into the embedding mold from the outer edge to the tip of the wing. The outer edge was always oriented closest to the identifying number on both the cassette and slide. Each bat’s left wing sections were stained with both PAS and H&E.

Histopathologic Examination of the Muzzles and Wings

Histopathology was done using a Nikon Eclipse 80i upright light microscope connected to a QImaging Retiga EXi color camera, and Dell desktop computer and monitor for imaging. Metamorph was the software program used to image the bats. Only living animals were analyzed because dead bats did not contribute any further usable data. Additionally, both time points of the 0.2% chitosan groups (3 and 6) were excluded from further analysis.

For the muzzles, the degree of fungal presence on or in the tissue was scored using another ‘0-3 scoring system’ from the PAS slides. The contrast between bat tissue and fungus was much clearer on this stain compared to the H&E. Fungal presence was defined as purple/dark magenta curved/round conidia or filamentous hyphae present on the surface or
within the epidermal tissue layer of bat muzzle. All sections (three or four) from each bat muzzle included were observed.

A score of '0' indicated no identifiable fungus was present throughout any slice. This could include individual spores that were scarcely scattered and countable, deeming them insignificant. A score of '1' indicated light or tiny groupings of fungus present on epidermal surface throughout any or all slices and no fungal penetration into tissue (epidermis) present throughout any or all slices. A score of '2' indicated heavy or large groupings of fungus present on epidermal surface throughout any or all slices and no fungal penetration into tissue (epidermis) present throughout any or all slices. A score of '3' indicated fungal penetration into tissue (epidermis) present throughout any or all slices. Bat muzzles were scored starting with a score of '0' and progressively moved up if fungus was present and depending on the degree of it. If no positive determination could be made for a specific spot of fungal presence, then it was not counted as significant. The scoring methodology used for the periodic acid Schiff-stained histology sections of the muzzles is described below. Images were taken of each included bat that represented its overall score based on the amount of fungal presence on or in the muzzle tissue (see Fig. 4).
The fungus stained dark purple and the surrounding tissue stained blue, turquoise, and light purple. All images were from different bats and all bars = 50 μm. Panels A and B (score of
‘0’) show no fungal presence on the surface (arrows) or penetration within the cutaneous tissue anywhere throughout the histology sections. Panels C and D (score of ‘1’) show light groupings of fungal presence on the surface (arrows) anywhere throughout the tissue samples, but no penetration within the cutaneous tissue anywhere throughout the histology sections. Panels E and F (score of ‘2’) show dense groupings of fungal presence on the surface (arrows) anywhere throughout the tissue samples, but no penetration within the cutaneous tissue anywhere throughout the histology sections. An empty hair follicle E has become heavily invaded with fungus (arrow), but penetration into the cutaneous tissue has not yet occurred. Panels G and H (score of ‘3’) show fungal penetration within the cutaneous tissue (arrows) anywhere throughout the histology sections. Finally, panel H shows densely aggregated fungal invasion into the underlying tissue (upper-right arrow).

For the wings, it was hypothesized that *Pd* caused an early inflammatory response after emergence from torpor and this was verified by the number of neutrophilic infiltrations observed in the wing tissue. These groups of neutrophils were counted throughout the wing sections using the H&E slides as neutrophils showed up much clearer on this stain compared to the PAS. Groupings consisted of six or more neutrophils in close proximity. If the morphology of a cell or group of cells could not be determined, then it was not included. Groupings that spanned a large area were considered as one focal accumulation of neutrophils. Images were taken that showed examples of focal accumulations of neutrophils in the wing tissue.

**Statistical Analyses**

All statistical analyses were conducted in either Microsoft Excel or IBM SPSS Statistics 23 software. A p-value ≤ 0.05 was considered statistically significant for all tests.
RESULTS

Survival

112 out of 180 bats total survived until the harvest day. The survival data among all groups is depicted in Table 1 and Figure 5 below.

Table 1. Survival among All Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Survivors</th>
<th>Total Male:Female</th>
<th>Male:Female Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: Clean Control</td>
<td>20</td>
<td>9:11</td>
<td>9:11</td>
</tr>
<tr>
<td>2: Infected Control</td>
<td>10</td>
<td>12:8</td>
<td>4:6</td>
</tr>
<tr>
<td>3: Active 0.2%</td>
<td>9</td>
<td>12:8</td>
<td>3:6</td>
</tr>
<tr>
<td>4: Active 0.1%</td>
<td>12</td>
<td>11:9</td>
<td>5:7</td>
</tr>
<tr>
<td>5: Active 0.05%</td>
<td>14</td>
<td>12:8</td>
<td>8:6</td>
</tr>
<tr>
<td>6: Early 0.2%</td>
<td>12</td>
<td>12:8</td>
<td>5:7</td>
</tr>
<tr>
<td>7: Early 0.1%</td>
<td>16</td>
<td>12:8</td>
<td>8:8</td>
</tr>
<tr>
<td>8: Early 0.05%</td>
<td>15</td>
<td>12:8</td>
<td>8:7</td>
</tr>
<tr>
<td>9: Early 1%</td>
<td>4</td>
<td>8:12</td>
<td>1:3</td>
</tr>
</tbody>
</table>
All 20 bats in the Clean Control group survived until the harvest when the experiment was ended and was the only group to have 100% survival. Only 50% of the bats in the Infected Control group survived until the harvest. The Early 0.1% and 0.05% groups had the highest survival among treatment groups. Interestingly, the Active 0.2% and Early 1% treatment groups had less overall survival than the Infected Control group. There was a beginning male:female ratio of 100:80, and at the end of the study the male:female survivor ratio was 51:61. Therefore, 51% of the males survived, while 76% of the females survived. The Early groups fared better than Active groups overall, albeit the Early 1% group had the worst survival among treated bats. The more dilute 0.1% and 0.05% groups had higher survival outcomes compared to the more viscous 1% and 0.2% groups.

The data portraying loss in body mass percentage between sexes among all groups of bats that survived until the harvest is shown in Table 2 below.
Table 2. Average Loss in Body Mass Percentage between Sexes among All Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Tot Avg % Mass Lost</th>
<th>M Avg % Mass Lost</th>
<th>F Avg % Mass Lost</th>
</tr>
</thead>
<tbody>
<tr>
<td>3: Active 0.2%</td>
<td>28.765 ± 5.841</td>
<td>29.136 ± 9.031</td>
<td>28.579 ± 3.209</td>
</tr>
<tr>
<td>4: Active 0.1%</td>
<td>27.633 ± 4.231</td>
<td>24.914 ± 3.045</td>
<td>29.575 ± 3.874</td>
</tr>
<tr>
<td>5: Active 0.05%</td>
<td>29.148 ± 4.449</td>
<td>28.323 ± 2.747</td>
<td>30.318 ± 5.832</td>
</tr>
<tr>
<td>6: Early 0.2%</td>
<td>29.260 ± 4.940</td>
<td>26.936 ± 4.376</td>
<td>30.921 ± 4.642</td>
</tr>
<tr>
<td>7: Early 0.1%</td>
<td>29.090 ± 3.930</td>
<td>27.062 ± 3.378</td>
<td>31.118 ± 3.354</td>
</tr>
<tr>
<td>8: Early 0.05%</td>
<td>26.419 ± 5.890</td>
<td>25.315 ± 7.090</td>
<td>27.681 ± 3.729</td>
</tr>
<tr>
<td>9: Early 1%</td>
<td>26.359 ± 2.827</td>
<td>22.572 ± 0.000</td>
<td>27.621 ± 2.068</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation.

The average percent mass lost refers to only the living animals. Dead animals were not included with these data.

While the survival data was important in gathering an overall idea of the treatment’s ability to have a positive impact on WNS, it did not tell a lot about the infection itself nor the degree to which it was at by the time each individual bat was harvested. Observing how the fungus relates to the host’s tissue, penetration into the epidermal tissue, and associated damage, is crucial for determining the effectiveness of chitosan on a given bat. Therefore, gross examination of the tissue was utilized to try to better understand the treatment’s effect on the fungus.
Gross Examination of the Wings: Digital Camera

The digital camera provided photos of the dorsal side of each living bat’s right wing. These photos were intended to be used to detect fungal and lesion presence, and the degree of tissue damage on the wing of each living bat. Additionally, the backlight images were intended for examination of the wing vasculature. The summary of the initial wing examination including digital photos using a backlit screen and UV light, and the Laser Doppler System scanner is described below. Figure 6 summarizes the digital images used to grossly examine the wings.
Figure 6. Gross Wing Examination Summary
All images used were of living bats and all bars = 1 cm. Panel A shows a digital photograph of a wing from an Infected Control bat using a backlit screen and showing no visible inflammation (arrow) or damage (arrowhead). Panel B shows a digital photograph of the same wing in panel A using UV light and showing no visible fungal presence or damage (arrow), and the small dots (arrowhead) were not designated as fungus. Panel C shows a digital photograph of a wing from a different Infected Control bat than in panel A using a backlit screen and showing damage (arrows) on one layer of the wing membrane. Panel D shows a digital photograph of the same wing in panel C using UV light and showing fungal presence or lesion fluorescence (arrows). Panel E shows a digital photograph of a wing from an Active 0.1% bat using a backlit screen and showing heavy damage (arrows) on one layer of the wing membrane and increased perfusion of the blood vessels as compared to those in panel A, and a possible indication of inflammation (arrowheads). Panel F shows a digital photograph of a wing from a different bat than in panels A through E stretched out before thermal imaging via the Laser Doppler System scanner. Finally, panels G and H show images of the same wing in panel F, but at different time points using the scanner and showing the amount of thermal activity present in the wing from bloodflow.

While viewing the backlight images, there were a couple of abnormalities on the wing membranes that were noted. Figure 7 below depicts dark focal pinpoint spots surrounded by a lighter halo (arrows).
These spots depicted above could be seen on some of the bats and were initially thought to be fungal colonies. However, this was ruled out because they did not have any UV fluorescence to confirm fungal presence and similar areas were found on the Clean Control group.

Figure 8 below depicts small, well-defined tears near the I.D. band (arrow) on some of the bats that were also noticed during the examination.
These holes were thought to have been caused by tension from the band itself and not from *Pd*. Consequently, they were not assumed to have been caused by the infection.

Other abnormalities included complete holes through the membrane that could not be determined if they were caused by *Pd*. They did not possess the same characteristics as the other tears that UV fluoresced. The lesions that did UV fluoresce had either full or partial tears with epithelial lipping and were grouped together rather than spread apart. Sometimes, UV fluorescence could be seen in areas that did not have a tear on the backlight images. Some of this fluorescence was likely caused by dust (seen as brighter and more speckled), but other areas that had a dull, yellowish appearance and no clear damage were likely attributed to fungal presence that had not yet led to a lesion. The fluorescence of dust appeared as a brighter, discrete, speckled pattern whereas the fungus seemed to have a slightly duller, yellow color with irregular
borders. Figure 6 above shows clear damage (panel C) to the wing with corresponding fluorescence (panel D) as was expected for infected individuals.

The majority of the UV light images of the wings were not clear enough to analyze further. Figure 9 below shows an example of how many of the UV images turned out.

Figure 9. Unclear UV Image of Wing

The wing depicted above is too saturated and unclear to make any claims about fungal presence. UV fluorescence could not be determined from these photos, but the digital photos using a backlit screen of white light were clear enough to view tears and holes (i.e. damage) in the wings. The UV pictures were used as tools to help confirm or deny questionable fungal presence as well as the affected area. Unfortunately, many of the UV pictures turned out blurry
despite consistent camera positioning and focusing. This may have been caused by the chitosan or temperature of the bat wing, which disrupted the imaging technique and outcome. Furthermore, many of the digital photos under UV light did not turn out well enough to be examined and analyzed. This meant it was difficult to match noticeable damage on the backlight images with fungus on the UV images. However, Figure 6 (B and D) above shows what the wing images should have looked like under optimal conditions.

A possible alternative to this would be to use a dark room that eliminates all outside sources of light. If the pictures still turn out unclear with a pink hue, then it is likely that the wing temperature could be the cause. In the current experiment, a curtain was used to shade the camera from any ambient light, but was not always replaced in the exact position as it was previously. Conversely, a sliding rotating door of a dark room could allow exact repositioning and complete elimination of ambient light. Also, during the harvest, bats had to be brought in and photographed quickly in order to get through all of them. Therefore, there was not a lot of time to constantly adjust the curtain every time a new bat was photographed.

After closely examining and scoring the degree of lesion presence on the digital photos taken using backlight and UV light of each living bat, there were significant differences found between the Clean Control group and all other groups regarding lesion scores of the wings.

There was no obvious pattern to the wing lesions, which is understandable, as the fungus most likely has no ordinary pattern of growing on the bats. After a blind scoring of the lesions, a trend between the experimental groups and significance was observed [Chi-square: \( \chi^2 = 46.87, \) df = 16, P-value = 7.124E-05]. However, this did not give a completely accurate answer as some of the data had to be left out because no scores of ‘3’ were recorded during the scoring procedure, but the test could not be ran with all values of ‘0’ in a column. Therefore, a Kruskal-Wallis one-
way analysis of variance test was ran which provided a more accurate representation of the data [Kruskal-Wallis: \( \chi^2 = 37.118, \text{df} = 8, \text{P-value} = 1.09\text{E}-05 \)]. After running this test across all groups and following up with pairwise post hoc tests, significant differences of lesions scores between the Clean Control and all other groups resulted. Table 3 below summarizes these results.

Table 3. Wing Lesion Scores

<table>
<thead>
<tr>
<th>Group</th>
<th>Score 0</th>
<th>Score 1</th>
<th>Score 2</th>
<th>Score 3</th>
<th>Average Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: Clean Control</td>
<td>19</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0.0500</td>
</tr>
<tr>
<td>2: Infected Control</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0.6000**</td>
</tr>
<tr>
<td>3: Active 0.2%</td>
<td>1</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>1.0000**</td>
</tr>
<tr>
<td>4: Active 0.1%</td>
<td>1</td>
<td>10</td>
<td>1</td>
<td>0</td>
<td>1.0000**</td>
</tr>
<tr>
<td>5: Active 0.05%</td>
<td>4</td>
<td>7</td>
<td>3</td>
<td>0</td>
<td>0.9286**</td>
</tr>
<tr>
<td>6: Early 0.2%</td>
<td>4</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0.6667**</td>
</tr>
<tr>
<td>7: Early 0.1%</td>
<td>3</td>
<td>12</td>
<td>1</td>
<td>0</td>
<td>0.8750**</td>
</tr>
<tr>
<td>8: Early 0.05%</td>
<td>9</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0.4000*</td>
</tr>
<tr>
<td>9: Early 1%</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1.0000**</td>
</tr>
</tbody>
</table>

* = p-value ≤ 0.02 and ** = p-value ≤ 0.005 when compared to the Clean Control group.

The table above shows the total number of each score recorded for each group. One member from Group 3 was not scored because it was never confirmed whether it had survived until the harvest day even though images of its wings were taken, but only living animals were to be included with this part of the analysis. Significantly different results were taken from the Kruskal-Wallis test and pairwise post hoc comparisons and their values are in bold. However, no groups besides the Clean Control were significantly different from the Infected Control group.
The proximal 1/3 of the plagiopatagium just distal to the 5th digit was the most affected area as the most damaged wings appeared to have lesions located in this region. However, there was no obvious trend across any of the groups in terms of an exact area of the wing with the highest damage from the infection. It is expected that the fungus should have the ability to spread to other areas of a body, especially one with less surface area as in the case of a bat.

It should not be assumed that a high degree of wing damage in infected bats was only caused by the disease. Assessing wing conditions in subsequent experiments before and after inoculation with *Pd* is a means of testing if there is a distinct way in which the disease damages the wing as opposed to other ways of acquiring injury. That is, a captured bat may have already endured damage to its wings from flying into trees and it would be necessary to rule out any possibilities from alternative forms of injury. Also, assessing infected wings before and after treatment with chitosan is critical in understanding how well it works as a wound-healer in the future.

After viewing the blood vessels from the backlight images, there was no clear correlation with dilated vasculature and wing damage. The heavily damaged wings appeared to have more dilated and erythematous vasculature. The color of the blood vessels ranged from light brown to red with increased redness more prominent in the heavily damaged wings. However, this was not always true in undamaged wings. That is, some wings had no distinct damage but increased redness of the vasculature. Figure 10 below depicts the color range observed during the examination.
Figure 10. Range of Vasculature Color in Wings
The intensity of the color gradually increases starting with a pale brown all the way to a bright red starting in panel A and ending in panel F. Panels A through F all received wing lesion scores of ‘0’, whereas panels G and H received wing lesion scores of ‘1’ and ‘2’, respectively. The amount of redness in the vasculature does not appear to depend on the degree of damage to the wing necessarily. The undamaged wings show multiple degrees of vasculature color intensity. Furthermore, it was noticed that the damaged wings had very red blood vessels indicating a possible inflammatory response occurring after emergence from torpor. While panels A through F did not have visible damage, this is not to say that fungus was also not present. The UV light images would have helped confirm this otherwise.

It could not be determined whether the vasculature was more dilated or erythematous around areas of damage, or if it was dilated and erythematous diffusely throughout the pictured wing by looking at the images alone without a more detailed magnification. The vessel diameter tapered in the peripheral regions of the wing. When magnifying the image in attempts to obtain a better view of the vessel, the image was too blurry to distinguish. This was made even more difficult when the vessel was not pink or red and there was shadowing around the vessel.

Five vasculature diameter measurements of each living bat’s main blood vessel of its wing in the Clean Control and Infected Control group were recorded using the Metamorph imaging program and averaged (see Fig. 6A (arrow) and 6E (arrowheads)). An ANOVA test [ANOVA: $F = 3.844$, F crit = 4.196, P-value = 0.060] and a Kruskal-Wallis test [Kruskal-Wallis: $\chi^2 = 3.254$, df = 1, P-value = 0.071] were ran and both determined that the vascular diameter averages between the two groups were not statistically significant. Figure 11 below summarizes these results.
The surviving animals of the Infected Control group depicted somewhat higher vasculature diameter means than the Clean Control group, but not enough to pursue any further measurements. To save time, the other groups were not included.

It was believed that wing damage did not necessarily correlate with increased vasculature, but rather fungal presence does as it was the foreign substance that an active bat’s immune system would respond to. After viewing the backlight images, it was noticed that many, heavily damaged wings did appear to also have a fairly red vasculature. If the UV light pictures had turned out clearer, then those would have been assessed to confirm the amount of fungus on each wing and compared with the degree of redness of the vasculature. Of the photos of damage-

![Vasculature Diameter Graph](image)

**Figure 11. Vasculature Diameter.** Error bars represent standard error of the mean.
free wings with a high degree of red blood vessels, it was believed that there was fungus present on those wings that may have not yet formed a lesion.

No significant difference was shown between the two groups that should have had a high difference in terms of vasculature diameter (Clean vs. Infected Controls). It was hypothesized that surviving bats should have increased vasculature activity. Therefore, a future alternative would be to use better imaging software that can accurately measure diminutive lengths such as ImageJ. Because the digital image had to be zoomed in so far in order to see the vessels closer, the quality was partially disrupted and may have led to inaccurate readings. It would be hypothesized in the future that the damage to the wings and state of the vasculature are dependent on the degree of fungal presence.

Gross Examination of the Wings: Laser-Doppler System Scanner

The thermal scanner gave an output of the amount of perfusion activity in each living bat’s right wing. These data were intended to be used to detect an increased blood supply as an indicator of early or acute inflammation in the wing of each living bat. Figure 6 (G and H) above summarizes the images used to examine the wings.

After closely examining the images taken under the thermal scanner of each living bat, there were significant differences found between some of the groups regarding perfusion means. Perfusion measurements of all living bats from all groups were recorded and averaged. An ANOVA test [ANOVA: F= 2.09, F crit = 2.03, P-value = 0.043] and a Kruskal-Wallis test [Kruskal-Wallis: $\chi^2 = 17.594$, df = 8, P-value = 0.024] were ran and both determined that the perfusion value differences between some of the groups were statistically significant. Albeit, the results of the ANOVA test say to reject the null hypothesis, the difference between variable F
and F-crit had a difference of only 0.07, so it barely rejected the null hypothesis. Figure 12 below summarizes these results.

Figure 12. Perfusion Comparison. * = p-value ≤ 0.02 and ** = p-value ≤ 0.004 when compared to the Infected Control group. Error bars represent standard error of the mean.

No clear trend across group perfusion measurements regarding concentration nor time point seemed apparent in the above graph. Significantly different results were taken from the Kruskal-Wallis test and pairwise post hoc comparisons.

The scanner gave outputs that were inconsistent and not significant for the overall study. It was believed that temperatures from both the table and holder’s hands could have interfered with the thermal scanner, which skewed the data. In multiple photos, the specimen holder’s hands could be seen, which meant there was a high likelihood that their own hands’ vasculature
activity was also scanned and interpreted with the bats’. If this was true, then many of the readings included data that was not the bats’ vasculature activity and many bats could have had inaccurate values. Additionally, the temperature of the table holding the scanner could have interfered with the readings, although it would be hard to tell without a thermometer that could give a constant output of ambient temperatures. Even after comparing some of the backlight images to the scanner data, there was no clear consistency between redness of the vasculature and perfusion means. Some wings with a very low degree of vasculature intensity had a high perfusion mean and vice versa.

With the outside interference, it was difficult to tell whether there truly was a difference in vasculature activity between the controls and treatment groups. The statistical tests did result in a significant difference between the Clean Controls and the Active 0.1% and Early 0.2% groups. However, these groups may have had more or less thermal interference leading to such results. It was expected that after emergence from torpor, bats with fungal infection would be able to initiate an immune response to fight it off, but only if they had enough energy leftover to do so. The increased perfusion activity within the vasculature would have been an indicator of this response. Some of the treatment groups had more average perfusion values, while others had less compared to the controls.

The controls groups did not significantly differ in perfusion means, so it was difficult to make any conclusions that the other groups were actually inflammation-free or not using these readings alone. That is, the Clean Controls should have had fairly low readings, while the Infected Controls should have had fairly high readings because they received no treatment and required an immune response to counter the fungal loads. This is assuming that any surviving Infected Control bats could even initiate a response with their limited energy reserves remaining.
If chitosan did limit fungal presence completely, then it would make sense that the treatment groups also had low activity because they would not need a large immune response to fend off the fungus. Conversely, if chitosan only partially limited fungal presence, but enough to keep the bats alive until normal arousal in springtime, then the bats would have high activity because they would be healthy enough to fight off the infection on their own. This specific area of the study opens new pathways for continuing research. Perhaps smaller dosages of chitosan lead to increased vasculature activity and larger dosages lead to decreased vasculature activity. With that in mind, the Laser-Doppler System scanner is a useful tool that may contribute more meaningful data for subsequent studies.

No meaningful data resulted from the gross examination of the tissue using the digital photos and the thermal scanner. Thus, it was determined that microscopic examination of the tissue was needed in order to visualize the fungus and analyze chitosan’s effect on it further.

Histopathologic Examination of the Muzzles

Only the wings were initially inoculated with *Pd* and/or treated with chitosan. Therefore, bats that were allowed to groom before entering torpor were able to portray how effective this treatment was. Assuming that the fungus and treatment combination spread to the muzzles, it was determined chitosan could limit the fungus in that location.

To determine if chitosan had any significant effects on *Pd* presence, the muzzle tissue from all living bats in each group except the two 0.2% groups were examined. Each bat's muzzle histology slide was scored based on its degree of fungal presence.

After gross examination of all the selected groups’ histology slides, it was clear that multiple groups differed from the Infected Control group in terms of fungal loads and
histopathology scores. However, not all bats exhibited the same histopathology within each group. The scores ranged from ‘0-3’ in multiple groups, while others were more consistent, such as the Clean Control group. There seemed to be an overall trend for each group though, such as 90% of the Infected Control group had at least some fungal presence as was expected. Also, a couple of other trends were noticed that seemed fairly consistent for most groups. First, the living bats had noticeably less fungus as compared to the dead bats. The dead bats were not scored because the fungus was expected to grow freely on these animals with or without treatment, and did not contribute any further meaningful data. Thus, once an organism dies, it no longer has any form of natural defense and the small amounts of fungus present on its skin will begin to decompose it. Second, the tissue sections that were closer to the muzzle had noticeably more fungus as well.

After close examination of the muzzle histopathology slides, a Kruskal-Wallis one-way analysis of variance test was used to test if any of the groups were significantly different from each other. A pairwise post hoc test was then used to determine where any significance was found between specific groups. A significant effect of chitosan was observed on experimentally treated bats in the laboratory. There was clearly less fungal presence and tissue damage on bats in the Clean Control group, on bats receiving a 0.1% chitosan while remaining active, and on bats receiving a 0.05% and 1% chitosan 30 days after entering hibernation relative to the Infected Control group based on histopathology scores. Mean scores included were as follows: Clean Control: 1.0, Active 0.1% Treatment: 0.92, Early 0.05% Treatment: 0.87, Early 1% Treatment: 0.25, Infected Control: 2.1 on a scale from ‘0-3’ with ‘0’ representing no fungal presence and ‘3’ representing fungal penetration into the tissue and damage; [Kruskal-Wallis: $\chi^2 = 14.234$, df = 6, P-value = 0.027]. Table 4 and Figure 13 below summarize these results.
Table 4. Muzzle Histopathology Scores

<table>
<thead>
<tr>
<th>Group</th>
<th>Score 0</th>
<th>Score 1</th>
<th>Score 2</th>
<th>Score 3</th>
<th>Average Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: Clean Control</td>
<td>6</td>
<td>11</td>
<td>0</td>
<td>3</td>
<td>1.0000</td>
</tr>
<tr>
<td>2: Infected Control</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>6</td>
<td>2.100</td>
</tr>
<tr>
<td>4: Active 0.1%</td>
<td>4</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>0.9166</td>
</tr>
<tr>
<td>5: Active 0.05%</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>8</td>
<td>1.8571</td>
</tr>
<tr>
<td>7: Early 0.1%</td>
<td>4</td>
<td>5</td>
<td>1</td>
<td>6</td>
<td>1.5625</td>
</tr>
<tr>
<td>8: Early 0.05%</td>
<td>10</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td>0.8666</td>
</tr>
<tr>
<td>9: Early 1%</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0.2500</td>
</tr>
</tbody>
</table>
Figure 13. Muzzle Histopathology Score Comparison. * = p-value ≤ 0.03 and ** = p-value ≤ 0.02 when compared to the Infected Control group. Error bars represent standard error of the mean.

Comparison of significant treatment groups with the Infected Control group using the PAS-stained histology sections of the muzzles is described below. Example images of score examples from specific groups that exhibited a significant difference in fungal loads or penetration relative to the Infected Control group were compared with each other in Figure 14 below.
Group 1:  
Clean Control

Group 2:  
Infected Control

Group 4:  
Active 0.1%

Group 8:  
Early 0.05%

Group 9:  
Early 1%

Figure 14. Muzzle Histopathology Group Comparisons Summary

Group 2 was significantly different from all other depicted groups above regarding muzzle histopathology scores. The fungus stained dark purple and the surrounding tissue stained
blue, turquoise, and light purple. All images were from different bats and represented score examples from each group, and all bars = 50 µm. Panels A through C show Group 1 examples with none (arrow in A) to very little fungal presence (arrows in B and C) present on/in tissue of all bats. Panels D through F show Group 2 examples with fungal presence (arrows) present on/in tissue of most bats. Panels G through I show Group 4 examples with none (arrow in G) to very little fungal presence (arrows in H and I) present on/in surface of most bats. Panels J through L show Group 8 examples with no fungal presence (arrows) present on/in tissue of most bats. Some bats did portray fungal penetration. Finally, panels M through O show Group 9 examples with no fungus (arrows) present on/in tissue of almost all bats.

The fungus was clearly visible in the Infected Control group images showing penetration (dark purple areas) into the tissue indicating severe damage to the epidermis. Comparatively, all other depicted groups had much less fungal presence and severity of damaged tissue. Bats in the Active 0.05% and Early 0.1% groups did not significantly differ from bats in the Infected Control group with respect to fungal presence (histopathology) scores.

Histopathologic Examination of the Wings

To determine if Pd caused an early inflammatory response, the wing tissue from all living bats in both control groups and the Early 0.1% group were examined. The Early 0.1% group exhibited the highest survival among the treatment groups. Neutrophils group together and large focal accumulations or infiltrations indicated an ongoing acute inflammatory reaction was present in the wing. The number of focal accumulations of neutrophils in each selected living bat's wing histology slide was searched for and quantified.
After gross examination of all the selected groups’ histology slides, it was clear that the Clean Control group had much fewer focal accumulations of neutrophils overall as compared to the other two groups. Most of the Clean Control bats had less than five focal accumulations of neutrophils whereas the other two groups had many more. Once again, several trends were noticed while examining the wing tissue. First, there was more inflammation on the longer sections of the tissue in general, and this was most likely because of the increased surface area of the wing section relative to the smaller ones, and not necessarily the location of it taken from the animals. Second, the neutrophils were grouped together where fungus was nearby on or within the wing tissue. This would be expected if the fungus was the cause of the activation of the acute inflammatory response. Consequently, there was clear evidence of acute inflammation in the histology sections of wing membrane from most of the Infected Control group and the Early 0.1% group. Conversely, there was little evidence of it in the Clean Control group as expected.

The number of neutrophilic infiltrations in the wings was high in the Early 0.1% group. There were two outliers in this group with very high numbers of neutrophilic infiltrations compared to the other bats in that group. Therefore, the count average was slightly skewed. Without the two outliers, the average count for the Early 0.1% group drops to 32.14, almost exactly that of the Infected Control group.

In summary, all living bats were brought out from torpor by warming them up, but not all bats exhibited acute inflammation. The Clean Controls showed virtually no acute inflammation within their wings, whereas the Infected Control and Early 0.1% bats showed a very clear difference from the Clean Control bats in wing morphology using histopathologic examination. Thus, confirming the hypothesis that the fungus is the cause of an acute inflammatory response in the living bats’ wings.
After close examination of the wing histopathology slides, another Kruskal-Wallis one-way analysis of variance test was used to test if any of the groups were significantly different from each other. Additionally, another pairwise post hoc test was then used to determine where any significance was found between specific groups. There was significantly less inflammation in the Clean Control relative to the Infected Control and Early 0.1% groups based on the number of focal accumulations of neutrophils. Mean quantities included were as follows: Clean Control: 2.05, Infected Control: 32.10, Early 0.1% Treatment: 41.94; [Kruskal-Wallis: $\chi^2 = 32.462$, df = 2, P-value = 8.93*10^{-8}]. Figure 15 below summarizes these results.

![Wing Histopathology FNA Count Comparison](image)

Figure 15. Wing Histopathology FNA Count Comparison. * = p-value ≤ 0.0005 when compared to the Clean Control group. Error bars represent standard error of the mean.
Comparison of groups from the H&E-stained histology sections of the wings to detect ongoing acute inflammation is described below. Example images from all three groups show the overall morphology of the wings and were compared with each other in Figure 16 below.

**Group 1:**
Clean Control

**Group 2:**
Infected Control

**Group 7:**
Early 0.1%

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Figure 16. Wing Histopathology Group Comparisons Summary
Group 1 was significantly different from the other two depicted groups regarding the number of focal accumulations of neutrophils. The multi-lobed nuclei of the neutrophils stained dark purple and the surrounding cytoplasm stained pink. All images were from different bats and represented focal neutrophilic accumulation examples from each group, and all bars = 50 μm. Panels A and B show Group 1 examples with very few numbers of neutrophilic infiltrations overall. The blood vessels (arrows) show no sign of neutrophil presence or inflammation and the surface of the wings (arrowheads) show no fungus. Panels C and D show Group 2 examples with high numbers of neutrophilic infiltrations overall. In panel C, the neutrophils begin to accumulate in small groups (arrows) and may eventually form a lesion (arrowhead) indicating inflammation caused by infection. In panel D, the neutrophils appear to accumulate in large groups and form larger infiltrations (arrows) in response to the fungal presence on the surface of the wing membrane (arrowheads). Panels E and F show Group 7 examples with high numbers of neutrophilic infiltrations overall. In panel E, neutrophils (arrow) are accumulating near the surface of the wing where fungus is present (arrowheads). In panel F, there is a focal accumulation of neutrophils in a thinner section of wing tissue with no indication of an advanced lesion present.

Neutrophils contained multi-lobed, purple-stained nuclei and pink-stained cytoplasm. They were distinct from other cell types because of these characteristics. The neutrophils were clearly visible in the Infected Control and Early 0.1% groups while similar areas of the wing in the Clean Control group consistently showed either very few or no neutrophils at all. Bats in the Early 0.1% group did not differ significantly from bats in the Infected Control group with respect to focal accumulations of neutrophils and corresponding acute inflammation.
It was deemed too difficult to quantify the amount of fungus on the wings because it could have been easily rubbed away while handling and produced inaccurate results. Scoring the wings was also deemed too difficult because it could not be easily determined if fungal presence was on top of or inside of the thin wing membrane. However, the presence of neutrophils in the Infected Control and Early 0.1% bat wings indicated that they were initiating an acute inflammatory response against the fungus.
This research represented a novel way to test chitosan as an agent to combat WNS in the laboratory. After analyzing the results, it was clear that chitosan had the ability to limit the presence of *Pd* on little brown bats in the laboratory, but more importantly it increased their survival outcome. This laboratory investigation provided similar results to what is known about *Pd* and WNS in the literature and field regarding the observations of fungal invasion into the living tissue of bats, bat mortality, and an early inflammatory response evident from large amounts of neutrophils in the wing tissue.

The current study demonstrated that less viscous chitosan provided for better survival outcomes in treated bats. The Early 1% group only had four survivors out of twenty. However, of these survivors, three had no visible fungal loads on the surface or in the tissue of the muzzle and one had a single spot infection. These data showed that this concentration was the most effective at limiting the spread of *Pd* on bat muzzles. The caveat to this was that it may have also caused physiological changes that reduced survival, such as caused the bats’ wings to stick together, but this remains uncertain. Based on the arousal data collected, this group did not have significantly lower torpor bout lengths compared to the other treatment groups; however, it did have the lowest overall. Therefore, it could be speculated that the 1% chitosan was too viscous and may have disrupted the bats while in torpor leading to more arousal events and death in this group. Stickiness of the wings caused by the 1% chitosan may have contributed to the high death outcome observed in this group. In comparison, the Early time points of 0.05% and 0.1% chitosan were the most effective amounts for bat survival. The Early 0.05% group had a 75%
survival outcome and significantly less fungal presence on or in the muzzle tissue compared to the Infected Control group. In addition, the Early 0.1% group had an 80% survival outcome.

The histopathologic examination of the wings provided evidence that *Pd* is the cause of early inflammation observed in the infected bats after emergence from torpor, similar to the wing analysis in Meteyer et al. (2012), which also portrayed severe neutrophilic inflammation in bats with *Pd*. The highly vascularized nature of bat wings may have played a role in the early response observed in this experimental infection. Future studies should examine the wings several days after the bats have emerged from torpor for determination of the nature of the end stage lesions as seen in WNS. It would be hypothesized that other inflammatory infiltrations of cells would be evident during lesion development in the laboratory setting.

The bat muzzles were also examined to gauge the extent of the inflammatory response. After grossly examining the muzzles, no evidence of inflammatory infiltrates was observed in the muzzle tissue of even the most heavily infected bats, which was also similar to the muzzle analyses in Meteyer et al. (2009) and Wibbelt et al. (2013). This may mean that the immune systems of these bats needed more time to initiate a response in the muzzles compared to the wings. In the current study, living bats only had a short period of time after emergence from torpor to initiate an immune response. Like the wings, further studies are needed that examine muzzles several days after the bats have emerged from torpor for any ensuing inflammatory responses.

Moore et al. (2013) looked at immunological responses in little brown bats infected with and without *Pd* and did not observe elevated amounts of infiltrating leukocytes in uninfected bats as compared to infected bats, similar to the current study’s results. This supports the idea that infected bats able to survive to emergence from hibernation will initiate an immune response.
against the fungus in attempt to fight it off naturally as portrayed in the present study. It is possible that the early inflammatory responses observed in the present study’s wing histopathology could have been from the hibernation period, but this is not certain because it was not tested. With this same idea in mind though, an inflammatory response while in a shallow torpor may also have contributed to host death in this study. In addition, periodic arousal from torpor is normal in bats and inflammation can occur during these arousals.

One of the major obstacles of fighting WNS is developing a suitable treatment and method for administering it. Chemical and biological agents (e.g. fungicides) are options that have been considered and *Pd* has been shown to be susceptible to treatment *in vitro* (Foley et al. 2011; Vonhof et al. 2014). Fungicides would negatively affect the microflora inside caves and delivering a treatment by hand to each bat would also be unfeasible. Additionally, while chitosan may work in the laboratory setting, no suitable methodology has yet been developed for use out in the field. However, aerosolizing is one way that chitosan could be administered to bat populations in the field for mass distribution by spraying throughout caves (Vonhof et al. 2014).

It has been suggested that *Pd* could remain inside infected caves even after all bats have left or been removed, and the fungus can continue to grow off substrates found in cave soil (Raudabaugh and Miller 2013; Reynolds and Barton 2014). Thus, despite chitosan’s ability to control fungal presence on the bats in the laboratory, it is uncertain if it will be enough for field trials in infected hibernacula. Anything entering an infected cave could potentially spread the fungus to other areas. Treating caves with chitosan in an aerosol form, as described above, may prevent both the bats and their habitat from being overtaken by *Pd*. However, this would need to be tested as caves also contain a complex ecosystem of other organisms besides bats.
Hoyt et al. (2015b) demonstrated the idea of *Pd* persisting long-term in a laboratory setting over a period that spanned more than five years. Their results suggested that *Pd* may be able survive outside of hibernacula on cave-exploring equipment surfaces if they are stored in cool and dry conditions. This is crucial for understanding how to control the spread of WNS because chitosan has not yet been tested on cave surfaces in regards to limiting the presence of *Pd.*

While these data support the use of chitosan in the laboratory setting, further investigations will be needed to ascertain the overall effectiveness of chitosan in the field. Studying its healing ability is a possible target for continued research in bats infected with *Pd.* WNS drains bats of their energy, which is necessary in order for them to heal their wings and survive. Therefore, the likelihood of the bats healing their wings after infection with WNS is rather low if they are already severely weakened and will be likely unable to forage for food. Fuller et al. (2011) explained how bats’ wings could heal over time even from severe injuries. 78% of the recaptured bats in their study showed at least some healing. Therefore, this is an idea for future studies in the field involving chitosan and WNS by recapturing treated bats and examining their wings for signs of healing, and comparing them to those recaptured without treatment.

On this same note, assessing wing damage is a key part of diagnosing and treating WNS. While chitosan helped to improve survival outcomes in the present study, it remains uncertain if it will be enough for helping bats heal their wings in time for foraging, as this was not examined. However, determining this will be beneficial for further evaluating chitosan’s wound-healing abilities and effectiveness. If treated bats with *Pd* can survive hibernation with limited fungal presence and heal their wings before they must forage for food, then this would help to further
argue the use of chitosan in the field. Finally, other possible future directions include: studying bats post-hibernation to learn more about lesion pathogenesis, inflammation development, and survival; improved chitosan delivery systems and dilution vehicles that reduce viscosity; and investigating chitosan efficacy in other bat species and hibernacula.

Recently, two more bats species, *Miniopterus schreibersii* and *Rhinolophus euryale*, have been confirmed to have WNS-associated skin lesions (Zukal et al. 2016). The present study did not observe the characteristic skin lesions in the wings, but this may have been because the bats were not given enough time to initiate a prolonged immune response and develop lesions after they were awoken from torpor. Thus, it is imperative to continue assessing chitosan as a suitable treatment method because many different types of bats in addition to *Myotis lucifugus* could face detrimental population declines otherwise.

There are other ways to help prevent bat population decline. For now, it is best to try to reduce disease transmission to more bats by closing off known clean caves to humans and prohibiting entry (Foley et al. 2011). This may help slow down the spread of *Pd* because fungal material can adhere to hair, clothing, and shoes, and humans are known inadvertent carriers (Coleman et al. 2015). Foley et al. (2011) also mentioned other ways to reduce spread, such as encouraging people to report new cases to officials. A study by Vonhof et al. (2015) examined the genetic analysis of *Myotis lucifugus* and estimated the risk of WNS spreading further west where more of that species resides. Based on what they found, the current pattern observed in the spread of WNS may not apply to the entire span of the little brown bat across North America, but this is uncertain in other bat species.

The overall significance and implication of this study was to provide further evidence that chitosan is an effective treatment option for combating WNS in little brown bats. The major
impacts that the results of this research exerted on the scientific field included: further
demonstrating that chitosan works as a \textit{Pd} limiter; demonstrating that the muzzle is just as
important as the wings when it comes to histopathologic examination; and demonstrating that
this disease and treatment can be successfully simulated in a laboratory setting.

In conclusion, this work is promising for helping to save bats, but further research will be
needed to continue improving the treatment methodology. This research is valuable to the
scientific field as it provides a promising solution towards someday helping to prevent high
mortality due to WNS in North American bat populations.
REFERENCES


Date: July 8, 2014

To: Maarten Vonhof, Principal Investigator

From: Lisa Baker, Chair

Re: IACUC Protocol Number 14-07-01

Your protocol entitled “Testing the Efficacy of Chitosan to Combat White-Nose Syndrome” has received approval from the Institutional Animal Care and Use Committee. The conditions and duration of this approval are specified in the Policies of Western Michigan University. You may now begin to implement the research as described in the application.

The Board wishes you success in the pursuit of your research goals.

Approval Termination: July 7, 2015